(57) Abstract

A novel β_2-adrenergic receptor subtype; DNA coding for the same; a recombinant vector having the aforementioned DNA; a host cell transformed by the aforementioned recombinant vector; a process for producing the aforementioned β_2-adrenergic receptor subtype by culturing the aforementioned host cell; a method and kit for screening agonists and/or antagonists of the aforementioned β_2-adrenergic receptor subtype; and a method of assaying the expression of the aforementioned β_2-adrenergic receptor subtype in cells or tissues.

The method of screening agonists of the novel β_2-adrenergic receptor subtype of the present invention is useful for the development of remedies for a certain type of asthmatic disease. Recombinant animals genetically engineered by using the DNA for the aforementioned β_2-adrenergic receptor subtype provide an effective means of studying the relationship between β_2-adrenergic receptors and asthmatic diseases.

[Standard list of country names and associated two-letter codes]

SPECIFICATION

A Novel β_2-Adrenergic Receptor Subtype and Applications Thereof

Technical Field

The present invention relates to a novel β_2-adrenergic receptor subtype, DNA that codes for it, a recombinant vector containing the aforementioned DNA, a host cell transformed by the aforementioned vector, and applications thereof.

Background of the Invention

The human β-adrenergic receptor currently is known to have three subtypes: β_1, β_2, and β_3. Of these, the β_2-adrenergic receptor (hereinafter abbreviated β_2-AR), which is present mainly in the smooth muscles of the trachea, uterus, and blood vessels, has a muscle-relaxing effect. As a result, β_2-AR stimulant drugs are used as remedies for bronchial asthma. Also, the imbalance between muscle-contracting and muscle-relaxing adrenergic receptors has been hypothesized as one possible cause of bronchial asthma.

On the other hand, the cDNA of human β_2-AR has been cloned already, and its base sequence and protein amino acid sequence also have been determined (Kobilka et al., *Proc. Natl. Acad. Sci. USA*, *84* : 46-50 (1987); sequence no. 3 of the sequence listing). Recently, by analyzing the DNA of the β_2-AR for asthmatic patients and normal subjects, [the inventors] investigated the cause-and-effect relationship between the aforementioned genetic polymorphism and asthma, and it became evident that β_2-AR polymorphism occurred with high frequency not only in asthmatic patients, but also in normal subjects (Reihsaus et al., *Am. J. Respir. Cell Mol. Biol.*, *8* : 334-339 (1993)). However, the existence and expression of β_2-AR subtype genes different from the known β_2-AR genes or their polymorphs was completely unknown.

Disclosure of the Invention

The purpose of the present invention is to provide a novel β_2-AR subtype having an amino acid sequence different from [that of] the commonly known human β_2-AR. Also, another purpose of the present invention is to provide a method and kit for screening agonists and/or antagonists of the aforementioned β_2-AR subtype, by using either the aforementioned novel β_2-AR subtype or a transformed cell that expresses it. Yet another

purpose of the present invention is to provide a method of assaying the expression of the aforementioned β_2-AR subtype by detecting part or all of the genes for the aforementioned novel β_2-AR subtype.

The inventors of the present invention conducted assiduous research to achieve the aforementioned goal. As a result, they discovered for the first time[1] that, in addition to the known β_2-AR mRNA, a novel mRNA that was homologous to but clearly different from the known β_2-AR was expressed in the A431 line of human epidermoid carcinoma cells (Dainippon Pharmaceutical, catalog no. 09-1555; hereinafter, abbreviated simply as A431 cell) and human heart tissue. Furthermore, by cloning its cDNA, they determined the base sequence as well as the amino acid sequence of the protein encoded by it. The inventors of the present invention further discovered that the protein encoded by the aforementioned novel cDNA has the physiological function of β_2-AR and identified that the aforementioned protein is a novel β_2-AR subtype. They also established a method of screening agonists and/or antagonists of the aforementioned β_2-AR subtype, by using animal cells transformed by a recombinant vector containing the novel β_2-AR subtype cDNA. Furthermore, they used the RT-PCR method to amplify part of the aforementioned novel β_2-AR subtype mRNA to develop a method of assaying the expression of the aforementioned β_2-AR subtype by detecting the obtained cDNA fragment, thereby perfecting the present invention.

That is, the present invention is as described hereinafter.

(1) A novel β_2-AR subtype having a Kd value for [^{125}I]-cyanopindolol of approximately 75 pM and actually having the amino acid sequence shown in sequence number 1 of the sequence listing, particularly a novel β_2-AR subtype derived from humans, and more particularly a novel β_2-AR subtype expressed in the A431 cell or the human heart.

(2) DNA having a base sequence that codes for the aforementioned β_2-AR, and preferably the aforementioned DNA having the base sequence shown by at least base numbers 101 to 1345, in the base sequence shown in sequence no. 2 of the sequence listing.

(3) A recombinant vector containing the aforementioned DNA and a host cell transformed by the aforementioned recombinant vector, particularly an animal cell, and more particularly a Chinese hamster ovary (CHO) cell.

[1] Translator's note: "...for the first time" is redundant.

(4) A method of producing the aforementioned novel β_2-AR subtype, characterized in that the novel β_2-AR subtype is collected from a culture obtained by culturing the aforementioned transformed cells.

(5) A method of screening agonists and/or antagonists of the aforementioned receptor, by using the aforementioned novel β_2-AR subtype, and particularly the aforementioned screening method such that the aforementioned novel β_2-AR is used in the form of the aforementioned transformed cell.

(6) A kit for screening the agonists and/or antagonists of the aforementioned receptor that contains the aforementioned novel β_2-AR subtype, and particularly the aforementioned screening kit such that the aforementioned novel β_2-AR subtype is used in the form of the aforementioned transformed cell. Also, the aforementioned screening kit that further contains a reagent containing a marker ligand as well reagents for cAMP assay.

(7) The aforementioned novel β_2-AR subtype characterized in that all or part of the DNA for the aforementioned novel β_2-AR subtype is detected.

Brief Explanation of the Drawings

Figure 1 shows the process of cloning the novel human β_2-AR subtype cDNA from the total RNA derived from A431 cells.

Figure 2 shows the base sequence of the novel human β_2-AR subtype cDNA derived from A431 cells (from the 5′ terminus to base 700) and the amino acid sequence of the aforementioned β_2-AR subtype (amino acids 1-200). The upper base sequence shows the bases in the base sequence of the known human β_2-AR subtype cDNA, that differ from the base sequence of the novel human β_2-AR subtype cDNA. The lower amino acid sequence shows the amino acids in the amino acid sequence of the known human β_2-AR subtype, that differ from the amino acid sequence of the novel human β_2-AR subtype.

Figure 3 shows the base sequence of the novel human β_2-AR subtype cDNA derived from A431 cells (from base 701 to the 3′ terminus) and the amino acid sequence of the aforementioned β_2-AR subtype (amino acids 201-415). The upper base sequence shows the bases in the base sequence of the known human β_2-AR subtype cDNA, that differ from the base sequence of the novel human β_2-AR subtype cDNA. The lower amino acid sequence shows the amino acids in the amino acid sequence of the known human β_2-AR

subtype, that differ from [the amino acid sequence of] the novel human β_2-AR[2] subtype. Also, an [ellipsis] (...) indicates that the corresponding base does not exist.

Figure 4 is the structural diagram of the animal cell expression vector pKCN1.

Figure 5 is the structural diagram of the animal cell expression plasmid pKREX20 that contains the novel human β_2-AR subtype cDNA derived from the A431 cell.

Figure 6 is a graph showing the results of the ligand binding test for the novel human β_2-AR subtype derived from the A431 cell, expressed in the CHO/pKREX20 cell.

Figure 7 shows the Scatchard plot based on the results of the ligand binding test for the novel human β_2-AR subtype derived from the A431 cell, expressed in the CHO/pKREX20 cell.

Figure 8 is a graph showing the results of the testing of cAMP accumulation in CHO/pKREX20 cells that express the novel human β_2-AR subtype derived from A431 cells.

Figure 9 is (a) an electrophoretogram showing the expression of the novel human β_2-AR subtype in A431 cells and human heart tissue and (b) the restriction enzyme map of the area amplified by RT-PCR.

Brief Explanation of the Invention

The novel β_2-AR subtype of the present invention has a Kd value for [^{125}I]-cyanopindolol of approximately 75 pM and actually has an amino acid sequence that is shown in sequence no. 1 of the sequence listing. As long as the three-dimensional structure of the aforementioned β_2-AR subtype and its physical and chemical properties remain unchanged, such an amino acid sequence is not particularly limited, so at least one amino acid may be displaced, deleted, inserted or added.

The Kd value for [^{125}I]-cyanopindolol of the novel β_2-AR subtype of the present invention is the dissociation constant of the binding of [^{125}I]-cyanopindolol and the aforementioned subtype, that was derived from a receptor binding test that used transformed Chinese hamster ovary (CHO) cells that express the aforementioned β_2-AR subtype.

Derivation of the novel β_2-AR subtype of the present invention is not particularly limited, as long as [it is from] animal cells or animal tissue, preferably mammalian cells or mammalian tissue, and more preferably human cultured cells or human tissue. Examples of cultured cells are A431 cells in the epidermoid carcinoma cell line, etc. Examples of the tissue are heart or cerebral cortex tissue, etc.

[2] Translator's note: In the Japanese patent, "AR" also has a subscripted "2," which is clearly a typo.

particularly hamster-derived cells (e.g., CHO, BHK), mouse-derived cells (e.g., COP, L, C127, Sp2/0, NS-1, NIH3, T3), rat-derived cells, monkey-derived cells (e.g., COS1, COS3, COS7, CV1, Velo), and human-derived cells (e.g., HeLa [cells], diploid fibroblast cells, myeloma cells, Namalwa cells).

The expression vector can be introduced into a host cell by using a conventional, commonly known method. When introducing [a vector] into a mammalian cell, examples include the calcium phosphate coprecipitation method, the protoplast fusion method, the microinjection method, the electroporation method, etc.

The novel β_2-AR subtype of the present invention can be produced by culturing the expression vector-containing transformed cells prepared as aforementioned. The culture medium preferably contains an organic or inorganic nitrogen source and a carbon source necessary for the growth of the host cells (transformants). Examples of carbon sources include glucose, dextran, soluble starch, and sucrose. [Examples of] nitrogen sources [include] ammonium salts, nitrates, amino acids, corn steep liquor, peptones, casein, meat extract, soybean cake, and potato starch extract. Also, [this] may contain other nutrients (e.g., inorganic salts (e.g., calcium chloride, sodium dihydrogen phosphate, magnesium chloride), vitamins, antibiotics (e.g., tetracycline, neomycin, kanamycin, ampicillin)), as desired.

Culturing is performed by a method known in this field. The culturing conditions (e.g., temperature, culture medium pH, and culturing time) are selected appropriately so that β_2-AR subtype proteins are produced in large quantities.

For example, when the host is an animal cell, examples of the usable culture medium include the minimum essential medium (MEM) containing approximately 5–20% fetal calf serum (FCS), Dulbecco's Modified Essential Medium (DMEM),[3] RPMI-1640 medium, 199 medium, etc. The pH of the culture medium preferably is approximately 6-8, and culturing normally is performed for approximately 15-72 hours at 30-40 °C. It also is possible to ventilate and agitate, as required.

The novel β_2-AR subtype of the present invention exists as transmembrane protein in the plasma membrane of the host cell. Consequently, the aforementioned proteins are acquired from a culture obtained by culturing as aforementioned, by means of the following method:

First, cells are recovered by subjecting a culture to a normal method of filtration or centrifugation, [this] is suspended in a buffer solution, and the membrane is solubilized

[3] Translator's note: The Japanese patent here and throughout has "Dulbecco's Modified Essential Medium", but a search of the on-line literature indicates that the correct expansion of DMEM is probably "Dulbecco's Modified Eagle's Medium".

by adding a surfactant to the appropriate concentration. Surfactants include sodium dodecyl sulfate (SDS), cetyltrimethylammonium bromide (CTAB), etc. However, these have a powerful protein denaturing effect, so to fold proteins so that they possess bioactivity, it is necessary to utilize dialysis, etc., to remove the excess [amount] of the aforementioned surfactant by replacing it with a buffer solution containing a gentle nonionic surfactant (e.g., Triton X-100) or to dilute [it] to the appropriate concentration. Next, it is possible to isolate and purify the novel β_2-AR subtype by appropriately [applying] a generally used method in the presence of the surfactant. Examples of [such a method] are a method that utilizes the solubility (e.g., salt precipitation, solvent precipitation), a method that utilizes the molecular weight difference (e.g., dialysis, ultrafiltration, gel filtration, SDS-PAGE), a method that utilizes the charge (e.g., ion-exchange chromatography, hydroxyapatite chromatography), a method that utilizes the specific affinity (e.g., affinity chromatography), a method of utilizing the hydrophobicity difference (e.g., reverse-phase liquid chromatography), and a method that utilizes the isoelectric point difference (e.g., isoelectric point electrophoresis).

By using the novel β_2-AR subtype of the present invention, it is possible to screen agonists and/or antagonists of the aforementioned β_2-AR subtype. The used novel β_2-AR subtype preferably is in the form of a transformed cell that expresses the aforementioned β_2-AR subtype. Examples are the following screening methods:

(1) Receptor binding test: A given quantity of the aforementioned transformed cells is pre-reacted for a given amount of time with various concentrations of test compounds, in a culture medium containing the appropriate buffer solution. Then, a known radioactive ligand (e.g., [^{125}I]-cyanopindolol) for the novel β_2-AR subtype is added at a specific concentration (e.g., the Kd value concentration), after which [this mixture] is reacted for a given amount of time. After the reaction is complete, the cells are collected and their radioactivity is measured. With the radioactivity resulting from the specific binding after the radioactive ligand is added by itself as 100%, the substitution curve is created by plotting the ratios of the specific binding of the radioactive ligand to the concentration of a test compound. From the substitution curve are read the concentrations (IC_{50}) of the test compounds that reduce by 50% the aforementioned binding ratio, and these values are evaluated as the apparent affinity for the novel β_2-AR subtype of the test compound. Furthermore, the radioactivity resulting from the nonspecific binding of a ligand can be compensated for by subtracting the radioactivity resulting from the further addition of an excessive amount of β-antagonist (e.g., (-)-alprenolol).

(2) cAMP accumulation test: A given quantity of the aforementioned transformed cells is reacted for a given amount of time with various concentrations of test compounds,

in the appropriate buffer solution containing an adenylate cyclase substrate and cofactors as well as a phosphodiesterase inhibitor (e.g., 3-isobutyl-1-methylxanthine). Then the amount of generated and accumulated cAMP is measured by means of an enzyme immunoassay (EIA) method, etc. The concentration–reaction curve is created with the amount of cAMP when the maximum concentration of test compound is added as 100% and the amount of cAMP when it is not added as 0%. The concentrations (EC_{50}) of test compounds that yield a 50% accumulation rate are computed and evaluated.

In the receptor binding test, of the test compounds having affinity for the novel β_2-AR subtype, those having a cAMP accumulation action in the cAMP accumulation test are antagonists of the aforementioned novel subtype. Those [lacking] the cAMP accumulation action can be considered to be antagonists.

The kit for screening agonists and/or antagonists of the novel β_2-AR subtype of the present invention are not particularly limited, as long as it contains the aforementioned β_2-AR subtype. The aforementioned proteins may be in any form as long as they have normal physiological activity, and they may be isolated and purified [proteins] or transformed cells that express the aforementioned proteins. The preferred example is a transformed animal cultured cell that expresses the aforementioned proteins, taking into consideration the protein stability and ease of handling. In this case, however, it is desirable to use a cell line with a uniform amount of expression per cell.

More preferably, the screening kit of the present invention is one containing the cAMP assay reagent used in the cAMP accumulation test as well as a reagent containing a marker ligand used in the receptor binding test. Examples of the marker ligand include [^{125}I]-cyanopindolol, [^{3}H]dihydroalprenolol, [^{3}H]carazolol, etc. Also, examples of the cAMP assay reagent include antibodies to cAMP and its detection reagents.

Other than those aforementioned, various reagents (e.g., buffer solution, culture medium) and apparatus (e.g., reaction container) used in the receptor binding test or the cAMP accumulation test also may be included.

In cells or tissues, the expression of the novel β_2-AR subtype of the present invention can be determined by using the difference between the base sequences of the cDNA of the aforementioned novel subtype and the cDNA of the known human β_2-AR subtype, and [by then] differentiating between the two. A concrete example of an assay method is discussed in detail in Embodiment 5 hereinafter.

Next, the present invention will be explained concretely with reference to embodiments and test examples. In no way, however, is the present invention limited thereto.

β_2-N1 has a sequence that is identical to a part (equivalent to bases no. 90–107 in sequence no. 3 of the sequence listing) of the 5' untranslated region of known human β_2-AR cDNA and it has the *Sse*8387I recognition region upstream thereof. β_2-C1 has the sequence complementary to a part (equivalent to bases no. 1466–1486 in sequence no. 3 of the sequence listing) of the 3' untranslated region of the aforementioned cDNA and it has the *Hind*III recognition region downstream thereof. In the case of the known human β_2-AR cDNA, these primers yielded approximately 1.4-kbp amplified fragments.

25 pmol of each of the aforementioned primers and 2 units of Taq DNA were added to a reverse-transcription reaction solution, sterilized distilled water was [added] to a total [volume] of 100 μL, and 30 cycles of the amplification reaction were performed under the following conditions, by using an automatic thermal cycler (Perkin-Elmer): (1) denaturation: 95 °C, 30 sec.; (2) annealing: 55 °C, 30 sec.; and (3) extension: 72 °C, 1 min. After the reaction completed, the reaction solution was digested by restriction enzymes *Sse*8387I and *Hind*III. [It] then was subjected to agarose gel electrophoresis, and a single band of approximately 1.4 kbp was detected.

3) cDNA cloning and sequencing: This band area of the gel was cut out, and the Spin Bind™ DNA Recovery System (FMC BioProducts) was used to purify and recover the cDNA fragments. The DNA Ligation Kit (Takarashuzo Co.) was used to insert the aforementioned fragments into the pUC119 vectors consumed by *Sse*8387I and *Hind*III. After [these] were introduced into *E. coli* bacteria HB101 by means of a normal method, the transformants were selected on LB agar containing 100 μg/mL ampicillin. The obtained transformants were cultured in liquid by using the LB culture medium, after which the alkali method was used to extract the plasmid DNA (pUCβ_2). Electrophoresis was used to confirm the cDNA insertion, after which the base sequence of the inserted part was determined by using the BcaBEST™ Dideoxy Sequencing Kit (Takarashuzo Co.). The aforementioned process is shown schematically in Figure 1.

Figures 2 and 3 show the complete base sequence of the cloned cDNA and the amino acid sequence of the protein encoded for by the aforementioned DNA. (However, the sequences shown by bases no. 100–83 *[sic]* in Figure 2 and bases no. 1280–1300 in Figure 3 are equivalent, respectively, to the sense and antisense primer portions used in

the PCR reaction, so parts of the bases in the aforementioned regions might differ from the conventional cDNA sequence.)

This cDNA has an overall length of 1400 bp and an open reading frame (ORF) that codes for 415 amino acids. ([This is] equivalent to bases no. 101–1345 in the base sequence of sequence no. 2 of the sequence listing.) As a result of a homology comparison with the known human β_2-AR cDNA (in the base sequence of sequence no. 3 of the sequence listing, the sequence represented by bases no. 90–1486 is the portion corresponding to the overall length of the cloned cDNA) the cloned cDNA was 96.6% homologous to the known human β_2-AR cDNA in the amino acid sequence. However, 41 base substitutions were present, of which 30 were within the ORF. Of these, 13 lead to amino acid substitution. (In Figure 2, the bases that differ from the base sequence of the novel cDNA in the base sequence of the known human β_2-AR cDNA are listed above the base sequence of the cloned cDNA, and the amino acids that differ from the amino acid sequence of the novel protein in the amino acid sequence of the known human β_2-AR are listed below the amino acid sequence of the protein coded for by the cloned cDNA.) Also, a part was identified in which five bases of six consecutive bases (TGCGAA) within the 5' untranslated region differ from the known human β_2-AR cDNA (CCCAGC). Furthermore, two amino acids (serine (Ser), asparagine (Asn)) not in the known human β_2-AR were inserted after the asparagine (Asn) in the 357 position.

Embodiment 2 Cloning of the cDNA of novel human β_2-AR subtype from human heart tissue

With mRNA (Clontech) derived from human heart tissue as the material, novel β_2-AR subtype cDNA was cloned by means of the method of Embodiment 1, and its base sequence was determined. As a result, compared with the [β_2-AR subtype cDNA] derived from A431 cells, the substitution of three bases (in Figure 2, the thymine (T) of base no. 238 was changed to adenine (A); and in Figure 3, the thymine (T) of base no. 823 was changed to cytosine (C) and the adenine (A) at 1143 was changed to guanine (G)) was found in the novel β_2-AR subtype cDNA derived from human heart tissue. Except for the one of these that caused an amino acid substitution (in Figure 2, the leucine (Leu) at position 80 was changed to isoleucine (Ile)), they were completely common.

Embodiment 3 Production of the novel β_2-AR subtype expression plasmid for anima cells

According to the strategies shown in Figures 4 and 5, an expression plasmid for animal cells, that functionally supports the novel human β_2-AR subtype is constructed.

1) *Sal*I was used to digest the expression vector pKCRH2 for animal cells (Mishina et al., *Nature*, *307* : 604-608 (1984)) having the *Hind*III recognition site between the SV40 promoter and the same polyadenylation signal, and the DNA Blunting Kit (Takarashuzo Co.) was used to blunt, after which the 3.67-kbp fragment (similarly blunted) containing the expression cassette of the neomycin phosphotransferase gene (*npt*II) obtained by using *Acc*I and *Aat*II to digest a different animal cell expression vector pSV2-neo (Southern and Berg, *J. Mol. Appl. Genet.*, *1*: 327-341 (1982)) was ligated to this, and a normal method was used to introduce [it] into *E. coli* HB101, and the transformant was selected on LB agar containing 100 μg/mL of ampicillin and 25 μg/mL of kanamycin. The plasmid DNA (pKCN0) was extracted from the obtained transformant, and part was electrophoresed to confirm the introduction of the neomycin phosphotransferase gene. Then *Hind*III was used to digest the aforementioned plasmid, and the synthetic adapter described hereinafter was inserted and a multicloning site was introduced. This was introduced into *E. coli* HB101, and the transformant was selected on LB agar containing 100 μg/mL ampicillin. A normal method was used to extract the plasmid DNA (pKCN1). The insertion of the multicloning site was confirmed by using *Dra*I and *Hind*III to digest the obtained plasmid DNA and [by] then electrophoresing to detect the band at the approximately 430 bp position. (For pKCN0, an approximately 380-bp band was obtained).

Synthetic adapter (sequence no. 6 of the sequence listing):

5' — AGCTCCTGCAGGCGCGCCGATATCTCGAGCGGCCGCGGTACCA — 3'
3' — GGACGTCCGCGCGGCTATAGAGCTCGCCGGCGCCATGGTTCGA — 5'

2) *Sse*8387I and *Hind*III were used to digest the plasmid pUCβ_2 that contained the novel β_2-AR subtype cDNA prepared in Example 1. 2% NuSieve™ 3:1 Agarose (Takarashuzo Co.) gel was used to electrophorese, and approximately 1.4-kbp fragments were recovered and purified. The aforementioned fragments and the 8.3-kbp fragments of pKCN1 digested by *Sse*8387I and *Hind*III were ligated, a normal method was used to introduce [them] into *E. coli* HB101, and the

transformant was selected on LB agar containing 100 μg/mL of ampicillin. After the obtained transformant was cultured, a normal method was used to extract the plasmid DNA. *Sse*8387I and *Hind*III were used to digest [this], after which [this] was electrophoresed to confirm the insertion of the novel β_2-AR subtype cDNA. The novel β_2-AR subtype expression plasmid for animal cells obtained as aforementioned was named pKREX20.

Embodiment 4 Production of the novel β_2-AR subtype high-expression CHO cell line

The calcium phosphate coprecipitation method was used to introduce the plasmid pKREX20 into Chinese hamster ovary cells CHO-K1 (ATCC : CCL-61), and it was cultured for 14 days in a DMEM culture medium (ICN Biomedicals) containing 10% fetal calf serum, 11.5 μg/mL proline, and 600 μg/mL G-418 (Life Technologies), under the conditions of 37 °C and 5% CO_2, in a CO_2 incubator (LNA-122D, Tabai Seisakusho). Then the transformed cell line was selected.

For 50 G-418 resistance clones, the culture medium was removed, after which the cells were peeled from the incubator by letting [them] stand for 10 min. at 37 °C in phosphate buffer saline (PBS) containing 0.5-mM ethylenediaminetetraacetic acid (EDTA). Next, the cells were collected by centrifugation and were suspended so that the cell density reached approximately 5×10^6 cells/mL, in a 10-mM tris-hydrochloric acid buffer (pH: 7.5) containing 1-mM EDTA. 20 μL of the aforementioned suspension and 1.5-nM [^{125}I]-cyanopindolol (CYP) were admixed into 200 μL of RPMI-1640 culture medium (ICN Biomedicals) containing 1% calf serum albumin, 0.1% NaN_3, and a 20-mM HEPES buffer solution (pH: 7.4), after which this was allowed to stand for 2 hours at 4 °C. By using the BioDot device (Bio-Rad Laboratories), [this] was filtered and washed in the GF/C glass filter (Whatman plc) that had been presoaked in 0.3% polyethyleneimine, and radioactivity on the filter was measured by using a γ-ray counter. The clone with the highest radioactivity was selected and named novel human β_2-AR subtype high-expression cell line CHO/pKREX20-58.

Test Example 1 Test of the affinity of radioactive ligands to the novel human β_2-AR subtype

The novel human β_2-AR subtype high-expression cell line CHO/pKREX20-58 obtained in Embodiment 4 was cultured for 3 days in DMEM culture medium containing

10% fetal calf serum, 11.5 μg/mL proline, and 200 μg/mL G-418, in a CO_2 incubator and under the conditions 37 °C and 5% CO_2, after which the culture medium was removed. Then the cells were peeled from the incubator by letting [them] stand for 10 min. at 37 °C in PBS containing 0.5-mM EDTA. Next, the cells were collected by centrifugation and were suspended so that the cell density reached approximately 5×10^6 cells/mL, in a 10-mM tris-hydrochloric acid buffer (pH: 7.5) containing 1-mM EDTA. 20 μL of the aforementioned suspension and various concentrations of [^{125}I]CYP were admixed into 200 μL of RPMI-1640 culture medium containing 1% calf serum albumin, 0.1% NaN_3, and a 20-mM HEPES buffer solution (pH: 7.4), after which this was allowed to stand for 4 hours at 4 °C (Reaction A). Separately, a sample was prepared by further adding (-)-alprenolol (100 μM), a β-receptor antagonist, to the aforementioned mixture, and [this] was reacted similarly for 4 hours at 4 °C (Reaction B). For each, the method of Embodiment 4 was used to measure the radioactivity.

The radioactivity in reactant B resulted from nonspecific binding, so the radioactivity of the [^{125}I]CYP bound specifically to the human β_2-AR on the CHO/pKREX20-58 cells was determined by means of the following equation:

[radioactivity resulting from specific binding] =

[radioactivity of reactant A] – [radioactivity of reactant B]

(Furthermore, similar tests of the CHO-KI cell line did not detect the expression of β_2-AR endogenous to the aforementioned cells.)

The radioactivity resulting from the specific binding at each [^{125}I]CYP concentration varied according to the Michaels-Menten equation (Figure 6). Consequently, it was confirmed that the novel human β_2-AR subtype expressed on the CHO/pKREX20-58 cells actually had ligand binding capacity.

Also, Figure 7 shows the plotted results (Scatchard plot), with F as the isolated [^{125}I]CYP concentration, with B as the receptor-bound [^{125}I]CYP concentration (i.e., the number of receptors to which [^{125}I]CYP is bound, per cell), with B/F as the vertical axis, and with B as the horizontal axis. Letting the number of receptors per cell be B_{max} and the dissociation constant be Kd, the straight line in Figure 7 is expressed by

$$B/F = (-1/Kd) \cdot (B - B_{max})$$

Consequently, Figure 7 shows that the number of novel human β_2-AR per CHO/pKREX20-58 cell line is approximately 18,000 per cell, and the Kd value is 75 pM. (In the case of a CHO cell transformed by means of a recombinant vector having known human β_2-AR cDNA, the number of β_2-AR per cell is approximately 47,000 per cell and the Kd is 32 pM.)

Test Example 2 cAMP accumulation test

The novel human β_2-AR subtype high-expression cell line CHO/pKREX20-58 was cultured and recovered as in Test Example 1, after which it was suspended so that the cell density reached 2×10^6 cells/mL in Hanks' balanced salt solution[5] (ICN Biopharmaceuticals) containing 1-mM ascorbic acid and 1-mM 3-isobutyl-1-methylxanthine. 100 μL of the aforementioned suspension and various concentrations of (-)-isoproterenol (IPT) were admixed into 500 μL of Hank's balanced salt solution, and [this] was reacted for 30 min. at 37 °C, after which it was boiled for 5 min. and the reaction was terminated. The cAMP EIA System (Amersham) was used to measure the amount of cAMP in the supernatant following centrifugation of the aforementioned reaction solution. Letting the amount of cAMP when 10^5-M (-)-IPT was added and when (-)-IPT was not added be 100% and 0%, respectively, the concentration producing a 50% cAMP accumulation rate (i.e., EC_{50}) was calculated from the concentration–reaction curve by means of the least-squares method. As a result, when CHO/pKREX20-58 was stimulated by means of (-)-IPT, the increase in the concentration-dependent cAMP quantity was evident (Figure 8), and the EC_{50} value was 43 nM. Consequently, it was confirmed that the novel human β_2-AR subtype expressed on the CHO/pKREX20-58 cells actually had cAMP accumulation activity (i.e., adenylate cyclase activation activity)

Embodiment 5 Assay of the expression of the novel β_2-AR subtype in human cells and tissues

1) With mRNA derived from A431 cells extracted according to the method of Embodiment 1 and mRNA derived from human heart tissue (Clontech) as templates, RT-PCR was performed according to the method of Embodiment 1, by using the following synthetic oligonucleotides [as] the sense primer and antisense primer, respectively.

Sense primer (β_2-N2):
5' — GGGAATGGCTACTCCAGCAAC — 3'
(sequence no. 7 of the sequence listing)

[5] Translator's note: The Japanese literally translates "Hanks' balanced buffer salt solution," but HBSS seems implied here.

Antisense primer (β_2-C2):

5' — CTGCTTTACAGCAGTGAGTC — 3'

(sequence no. 8 of the sequence listing)

β_2-N2 has a sequence homologous to the novel human β_2-AR subtype cDNA (equivalent to bases no. 1151–1171 in the base sequence of sequence no. 2 of the sequence listing) and to the known human β_2-AR subtype cDNA (equivalent to bases no. 1240–1260 in the base sequence of sequence no. 3 of the sequence listing). Also, β_2-C2 has a sequence homologous to the novel human β_2-AR subtype cDNA (equivalent to bases no. 1334–1353 in the base sequence of sequence no. 2 of the sequence listing) and to the known human β_2-AR subtype cDNA (equivalent to bases no. 1417–1436 in the base sequence of sequence no. 3 of the sequence listing). Consequently, when these primers are used, it is possible to obtain amplified fragments of 203 bp from the novel human β_2-AR mRNA and of 197 bp from the known human β_2-AR mRNA.

2) The RT-PCR reaction solution obtained in the aforementioned 1) was divided in two, and one was digested by *Eco*RV; the other, by *Pma*CI. Then these were separated by means of 2% agarose gel electrophoresis. As a result of dyeing the aforementioned agarose gel with ethidium bromide, a band pattern such as the one shown in Figure 9(a) was obtained. That is, when the RT-PCR reaction products derived from the A431 cells were treated with *Eco*RV, thick bands of 151 and 52 bp and a thin band of 46 bp were observed. When [they] were treated with *Pma*CI, a thick band of 203 bp and thin bands of 146 and 51 bp were observed. On the other hand, when the RT-PCR reaction products derived from human heart tissue were treated with *Eco*RV, thick bands of 151 and 46 bp and a thin band of 52 bp were observed. When [they] were treated with *Pma*CI, contrary to the case of the A431 cells, a thin band of 203 bp and thick bands of 146 and 51 bp were observed.

As shown in Figure 9(b), the 197-bp amplified fragments derived from known human β_2-AR subtype cDNA were cut into fragments of 151 bp and 46 bp, and the 203-bp amplified fragments derived from the novel human β_2-AR subtype cDNA were cut into fragments of 151 bp and 52 bp. On the other hand, after treatment with *Pma*CI, the 197-bp amplified fragments derived from known human β_2-AR subtype cDNA were cut into fragments of 146 bp and 51 bp. By contrast, the base indicated by base no. 1208 in sequence no. 2 of the sequence listing mutated into cytosine (C) in the novel human β_2-AR subtype cDNA, resulting in the disappearance of the *Pma*CI recognition site (equivalent to bases no. 1288–1293 in

the base sequence of sequence no. 3 of the sequence list) present in the known human β_2-AR cDNA. So, the 203-bp amplified fragments derived from novel human β_2-AR subtype cDNA were not cut by *Pma*CI.

From the results aforementioned, for both the A431 cells and the human heart tissue, both the novel β_2-AR and known β_2-AR subtypes were expressed. However, it was confirmed that, in the A431 cells, the novel subtype was expressed in larger quantities, and in the human heart tissue, the known subtype was express in larger quantities.

Regarding the novel β_2-AR subtype of the present invention, glycine was substituted for the arginine at position 16 of the known human β_2-AR. Reihsaus et al. reported that asthmatic patients with DNA polymorphism that induces this substitution exhibit a specific clinical profile (*Am. J. Respir. Cell Mol. Biol.*, *8* : 334-339 (1993)). Consequently, the method [used] to screen the agonists of the aforementioned subtypes, that uses the novel β_2-AR subtype of the present invention is extremely useful for the development and study of asthma remedies with, as the active components, agonists such that the aforementioned subtypes exhibit singularly high sensitivity, particularly remedies for asthma patients in whom mainly the novel β_2-AR subtype of the present invention is expressed in the smooth muscles of the bronchial tubes.

Also, the method of assaying the expression of the novel human β2-AR subtype of the present invention is effective in the diagnosis of asthmatic patients who exhibit the aforementioned specific clinical profile.

Furthermore, as research materials for the elucidation of the relationship of β_2-AR to asthma, it is very useful to use the novel human β2-AR subtype DNA of the present invention to create test animals with the endogenous β2-AR knocked out by means of gene disruption as well as transgenic test animals into which novel human β2-AR subtype DNA was functionally inserted.

The present application is based on 1996 patent application no. 72914 that was applied for in Japan, and its entire contents are included in this specification. Also, the contents described in the publication mentioned here are included in this specification to such an extent that they are all specified herein.

[The translation stops here at the end of page 20 of the Japanese text and resumes with the claims starting on page 33 of the Japanese text.]

Claims

1. A β_2-adrenergic receptor subtype having a Kd value for [^{125}I]-cyanopindolol of approximately 75 pM and actually having the amino acid sequence shown in sequence no. 1 of the sequence listing.

2. The β_2-adrenergic receptor subtype of Claim 1, that is derived from humans.

3. The β_2-adrenergic receptor subtype of Claim 2, that is derived from A431 cells or the human heart.

4. DNA having a base sequence that codes for the β_2-adrenergic receptor subtype of any of Claims 1-3.

5. The DNA of Claim 4 having the base sequence shown by bases no. 101 to 1345, in the base sequence shown in sequence no. 2 of the sequence listing.

6. A recombinant vector containing the DNA of Claim 4 or 5.

7. A host cell transformed by the recombinant vector of Claim 6.

8. The host cell of Claim 7, such that the host cell is an animal cell.

9. The host cell of Claim 8, such that the animal cell is a CHO cell.

10. A method of producing a β_2-adrenergic receptor subtype, that includes the culturing of any host cell of Claims 7-9 and the collection of the β_2-adrenergic receptor subtype from the obtained culture, and that has the following properties:
    (i) Kd value for [^{125}I]-cyanopindolol: approximately 75 pM
    (ii) [It] actually has the amino acid sequence shown in sequence no. 1 of the sequence listing.

11. A method of screening agonists or antagonists of the aforementioned β_2-adrenergic receptor subtype, that uses any β_2-adrenergic receptor subtype of Claims 1-3.

12. A method of screening agonists or antagonists of the β_2-adrenergic receptor subtype, that uses any host cell of Claims 7-9.

13. A kit for screening agonists or antagonists of the aforementioned β_2-adrenergic receptor subtype, that contains any β_2-adrenergic receptor subtype of Claims 1-3.

14. A kit for screening agonists or antagonists of the aforementioned β_2-adrenergic receptor subtype, that contains any host cell of Claims 7-9.

15. The screening kit of Claims 13 or 14, that further contains a cAMP assay reagent or reagent containing a marker ligand.

16. A method of assaying the expression of a β_2-adrenergic receptor subtype, that includes the detection of all or part of the DNA of Claim 4 or 5 and that has the following properties:
    (i) Kd value for [^{125}I]-cyanopindolol: approximately 75 pM
    (ii) [It] actually has the amino acid sequence shown in sequence no. 1 of the sequence listing.

Figure 1

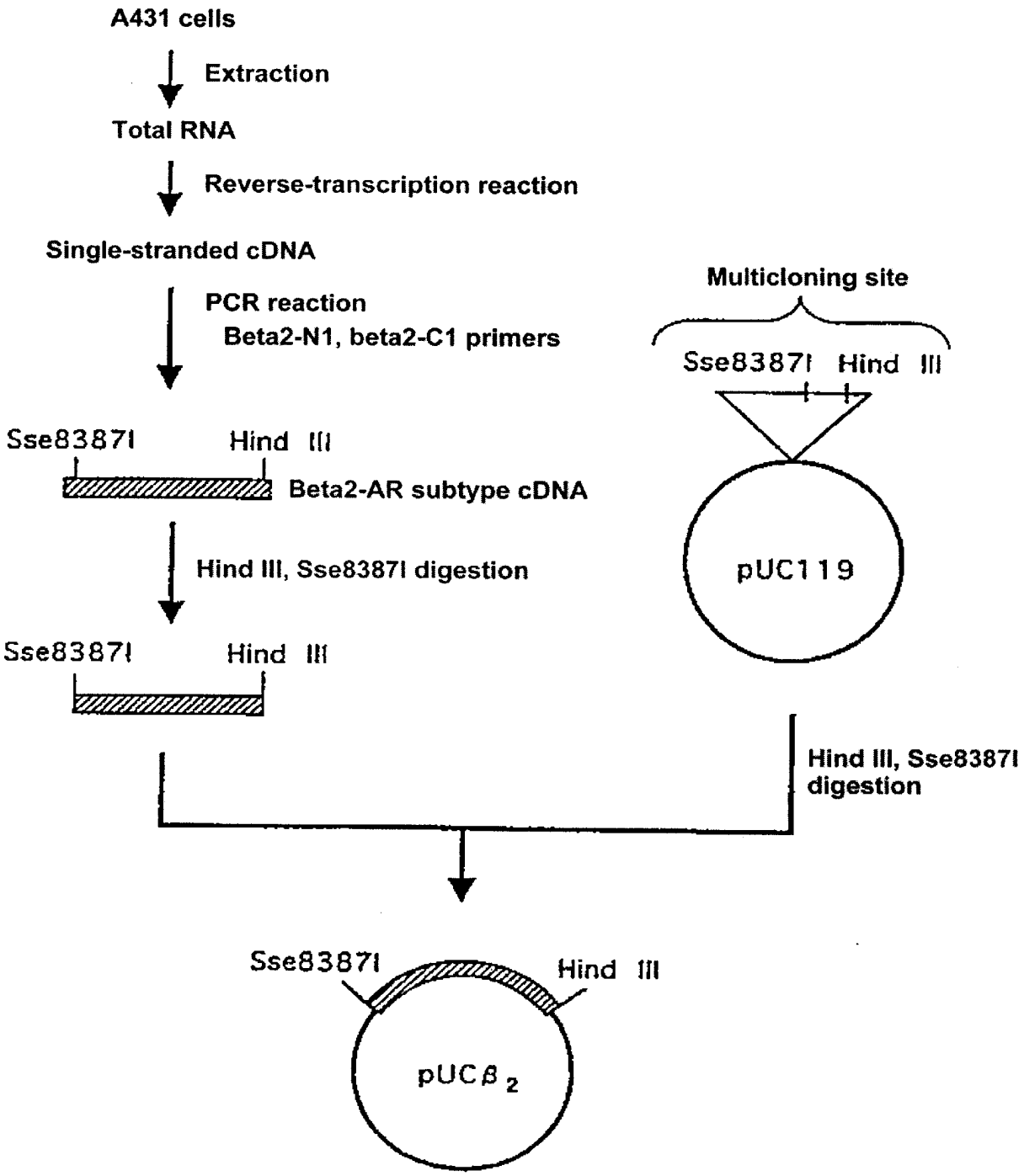

A431 cells
Extraction
Total RNA
Reverse-transcription reaction
Single-stranded cDNA
PCR reaction
Beta2-N1, beta2-C1 primers
Sse8387I
Hind III
Beta2-AR subtype cDNA
Hind III, Sse8387I digestion
Sse8387I
Hind III
Multicloning site
Sse8387I
Hind III
pUC119
Hind III, Sse8387I digestion
Sse8387I
Hind III
pUCβ2

Figure 2

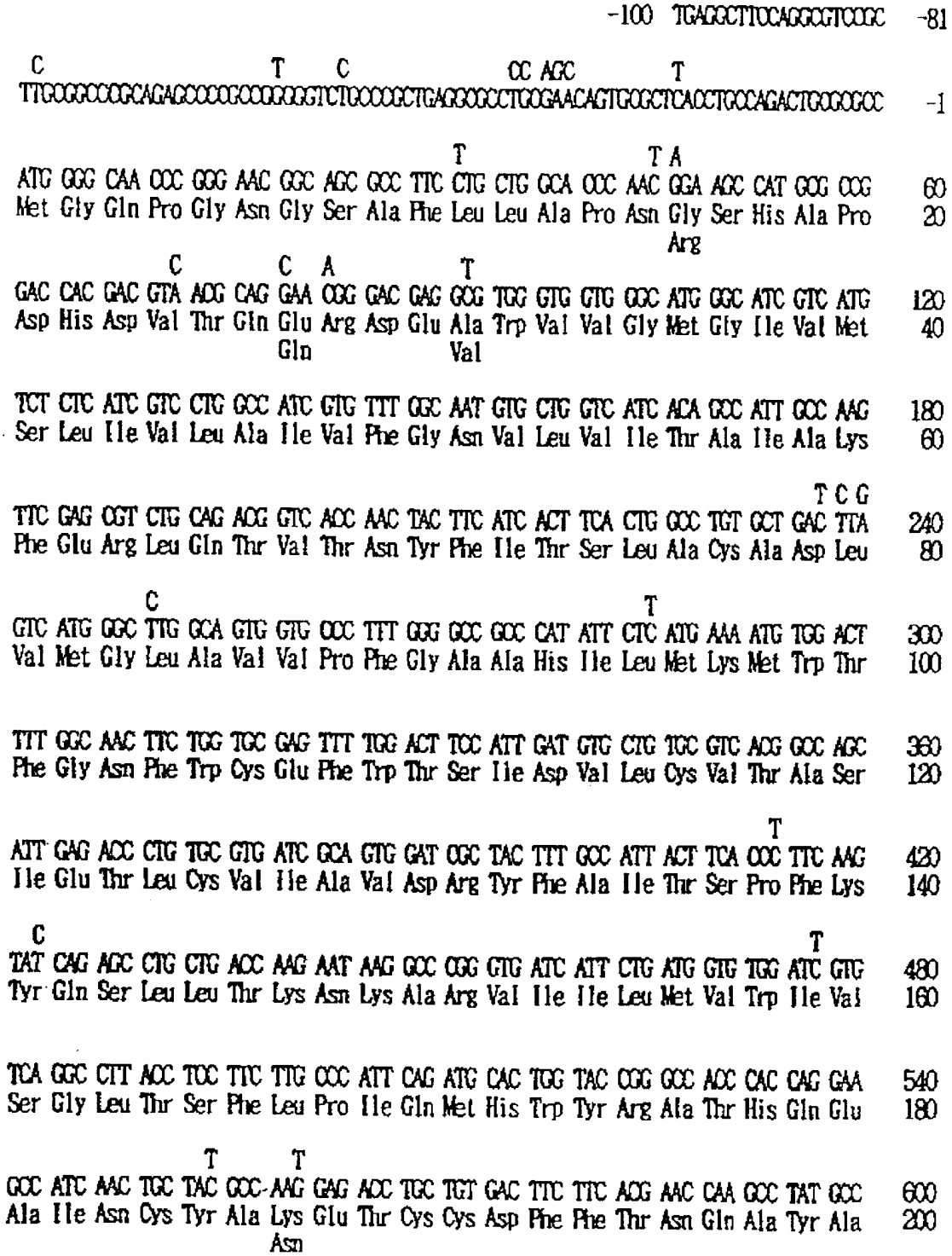

-100 TGAGGCTTCCAGGCGTCCCC -81
C T C CC AGC T
TTCGGGCCCGCAGAGCCCCGCCCGGGGTCTGCCCGCTGAGGCGCCTGCGAACAGTGCGCTCACCTGCCAGACTGCGCGCC -1
T T A
ATG GGG CAA CCC GGG AAC GGC AGC GCC TTC CTG CTG GCA CCC AAC GGA AGC CAT GCG CCG 60
Met Gly Gln Pro Gly Asn Gly Ser Ala Phe Leu Leu Ala Pro Asn Gly Ser His Ala Pro 20
Arg
C C A T
GAC CAC GAC GTA ACG CAG GAA CGG GAC GAG GCG TGG GTG GTG GGC ATG GGC ATC GTC ATG 120
Asp His Asp Val Thr Gln Glu Arg Asp Glu Ala Trp Val Val Gly Met Gly Ile Val Met 40
Gln Val
TCT CTC ATC GTC CTG GCC ATC GTG TTT GGC AAT GTG CTG GTC ATC ACA GCC ATT GCC AAG 180
Ser Leu Ile Val Leu Ala Ile Val Phe Gly Asn Val Leu Val Ile Thr Ala Ile Ala Lys 60
T C G
TTC GAG CGT CTG CAG ACG GTC ACC AAC TAC TTC ATC ACT TCA CTG GCC TGT GCT GAC TTA 240
Phe Glu Arg Leu Gln Thr Val Thr Asn Tyr Phe Ile Thr Ser Leu Ala Cys Ala Asp Leu 80
C T
GTC ATG GGC TTG GCA GTG GTG CCC TTT GGG GCC GCC CAT ATT CTC ATG AAA ATG TGG ACT 300
Val Met Gly Leu Ala Val Val Pro Phe Gly Ala Ala His Ile Leu Met Lys Met Trp Thr 100
TTT GGC AAC TTC TGG TGC GAG TTT TGG ACT TCC ATT GAT GTG CTG TGC GTC ACG GCC AGC 360
Phe Gly Asn Phe Trp Cys Glu Phe Trp Thr Ser Ile Asp Val Leu Cys Val Thr Ala Ser 120
T
ATT GAG ACC CTG TGC GTG ATC GCA GTG GAT CGC TAC TTT GCC ATT ACT TCA CCC TTC AAG 420
Ile Glu Thr Leu Cys Val Ile Ala Val Asp Arg Tyr Phe Ala Ile Thr Ser Pro Phe Lys 140
C T
TAT CAG AGC CTG CTG ACC AAG AAT AAG GCC CGG GTG ATC ATT CTG ATG GTG TGG ATC GTG 480
Tyr Gln Ser Leu Leu Thr Lys Asn Lys Ala Arg Val Ile Ile Leu Met Val Trp Ile Val 160
TCA GGC CTT ACC TCC TTC TTG CCC ATT CAG ATG CAC TGG TAC CGG GCC ACC CAC CAG GAA 540
Ser Gly Leu Thr Ser Phe Leu Pro Ile Gln Met His Trp Tyr Arg Ala Thr His Gln Glu 180
T T
GCC ATC AAC TGC TAC GCC AAG GAG ACC TGC TGT GAC TTC TTC ACG AAC CAA GCC TAT GCC 600
Ala Ile Asn Cys Tyr Ala Lys Glu Thr Cys Cys Asp Phe Phe Thr Asn Gln Ala Tyr Ala 200
Asn

Figure 3

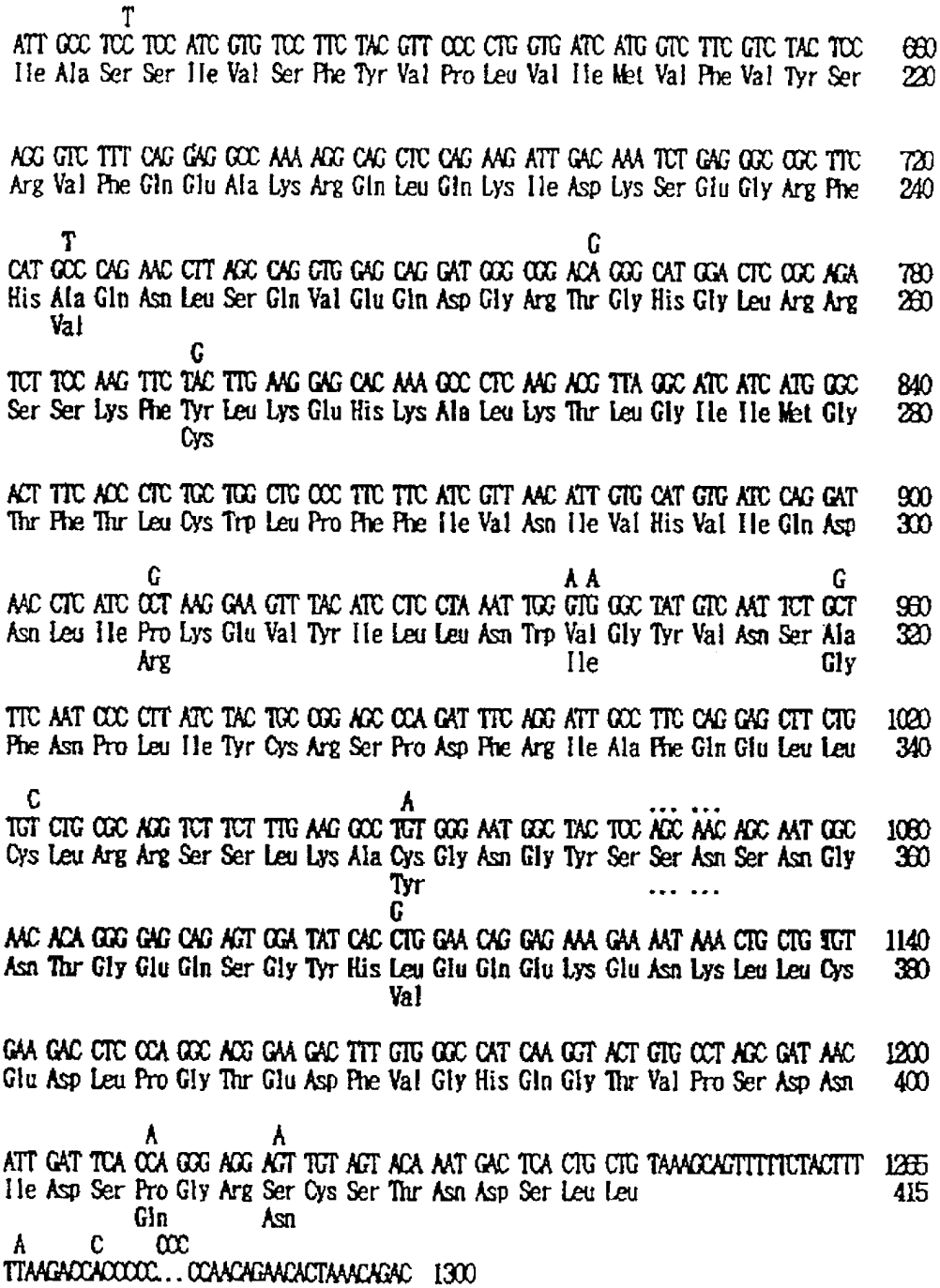

ATT GCC TCC TCC ATC GTG TCC TTC TAC GTT CCC CTG GTG ATC ATG GTC TTC GTC TAC TCC 660
Ile Ala Ser Ser Ile Val Ser Phe Tyr Val Pro Leu Val Ile Met Val Phe Val Tyr Ser 220
ACG GTC TTT CAG GAG GCC AAA AGG CAG CTC CAG AAG ATT GAC AAA TCT GAG GGC CGC TTC 720
Arg Val Phe Gln Glu Ala Lys Arg Gln Leu Gln Lys Ile Asp Lys Ser Glu Gly Arg Phe 240
CAT GCC CAG AAC CTT AGC CAG GTG GAG CAG GAT GGG CGG ACA GGG CAT GGA CTC CGC AGA 780
His Ala Gln Asn Leu Ser Gln Val Glu Gln Asp Gly Arg Thr Gly His Gly Leu Arg Arg 260
Val
TCT TCC AAG TTC TAC TTG AAG GAG CAC AAA GCC CTC AAG ACG TTA GGC ATC ATC ATG GGC 840
Ser Ser Lys Phe Tyr Leu Lys Glu His Lys Ala Leu Lys Thr Leu Gly Ile Ile Met Gly 280
Cys
ACT TTC ACC CTC TGC TGG CTG CCC TTC TTC ATC GTT AAC ATT GTG CAT GTG ATC CAG GAT 900
Thr Phe Thr Leu Cys Trp Leu Pro Phe Phe Ile Val Asn Ile Val His Val Ile Gln Asp 300
AAC CTC ATC CCT AAG GAA GTT TAC ATC CTC CTA AAT TGG GTG GGC TAT GTC AAT TCT GCT 960
Asn Leu Ile Pro Lys Glu Val Tyr Ile Leu Leu Asn Trp Val Gly Tyr Val Asn Ser Ala 320
Arg Ile Gly
TTC AAT CCC CTT ATC TAC TGC CGG AGC CCA GAT TTC AGG ATT GCC TTC CAG GAG CTT CTG 1020
Phe Asn Pro Leu Ile Tyr Cys Arg Ser Pro Asp Phe Arg Ile Ala Phe Gln Glu Leu Leu 340
TGT CTG CGC AGG TCT TCT TTG AAG GCC TGT GGG AAT GGC TAC TCC AGC AAC AGC AAT GGC 1080
Cys Leu Arg Arg Ser Ser Leu Lys Ala Cys Gly Asn Gly Tyr Ser Ser Asn Ser Asn Gly 360
Tyr
AAC ACA GGG GAG CAG AGT GGA TAT CAC CTG GAA CAG GAG AAA GAA AAT AAA CTG CTG TGT 1140
Asn Thr Gly Glu Gln Ser Gly Tyr His Leu Glu Gln Glu Lys Glu Asn Lys Leu Leu Cys 380
Val
GAA GAC CTC CCA GGC ACG GAA GAC TTT GTG GGC CAT CAA GGT ACT GTG CCT AGC GAT AAC 1200
Glu Asp Leu Pro Gly Thr Glu Asp Phe Val Gly His Gln Gly Thr Val Pro Ser Asp Asn 400
ATT GAT TCA CCA GGG AGG AGT TGT AGT ACA AAT GAC TCA CTG CTG TAAAGCAGTTTTTTCTACTTT 1265
Ile Asp Ser Pro Gly Arg Ser Cys Ser Thr Asn Asp Ser Leu Leu 415
Gln Asn
TTAAGACCACCCCC...CCAACAGAACACTAAACAGAC 1300

Figure 4

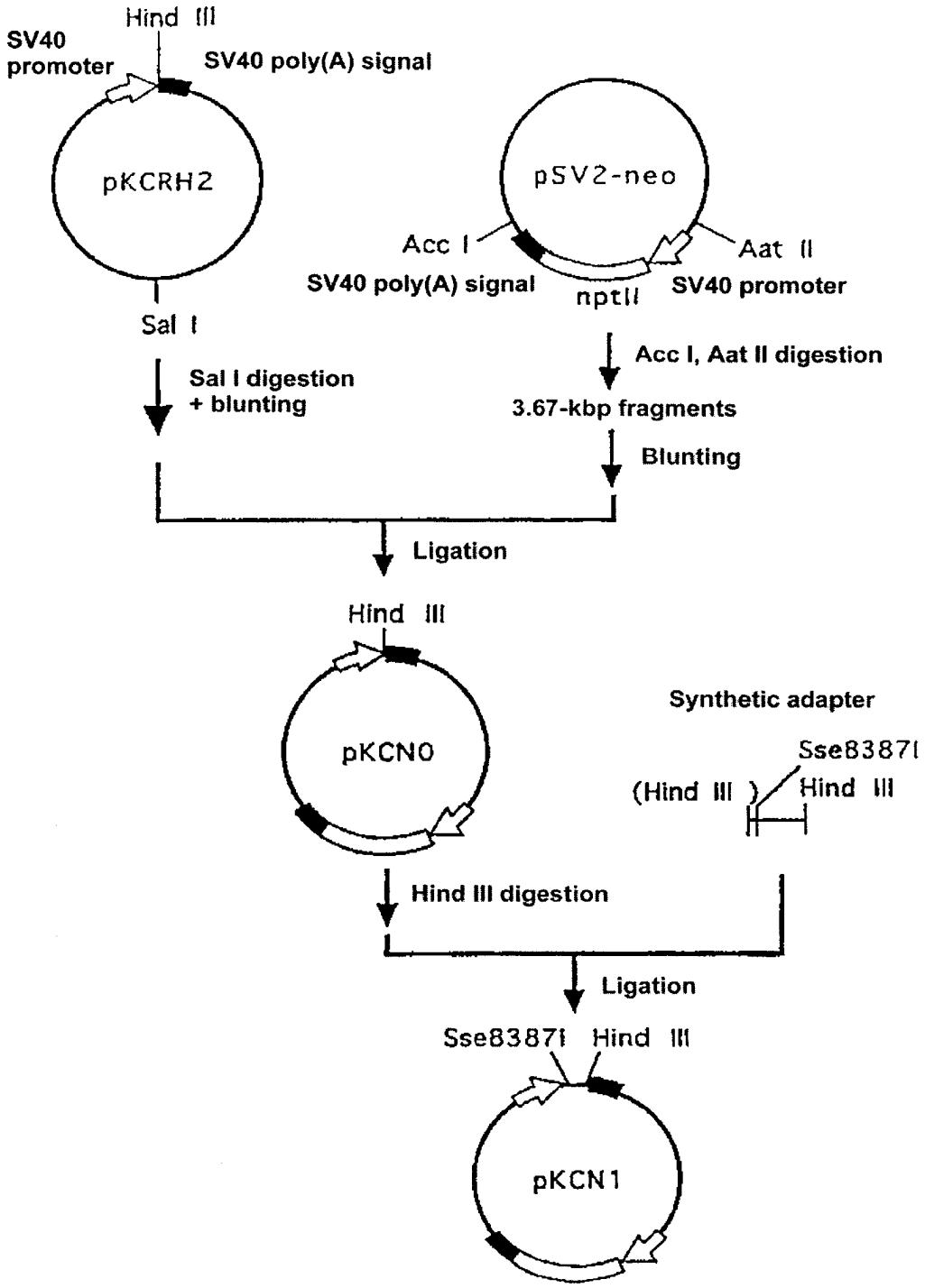

Hind III
SV40 promoter
SV40 poly(A) signal
pKCRH2
Sal I
Sal I digestion + blunting
pSV2-neo
Acc I
Aat II
SV40 poly(A) signal
nptII
SV40 promoter
Acc I, Aat II digestion
3.67-kbp fragments
Blunting
Ligation
Hind III
pKCN0
Synthetic adapter
Sse8387I
(Hind III)
Hind III
Hind III digestion
Ligation
Sse8387I Hind III
pKCN1

Figure 5

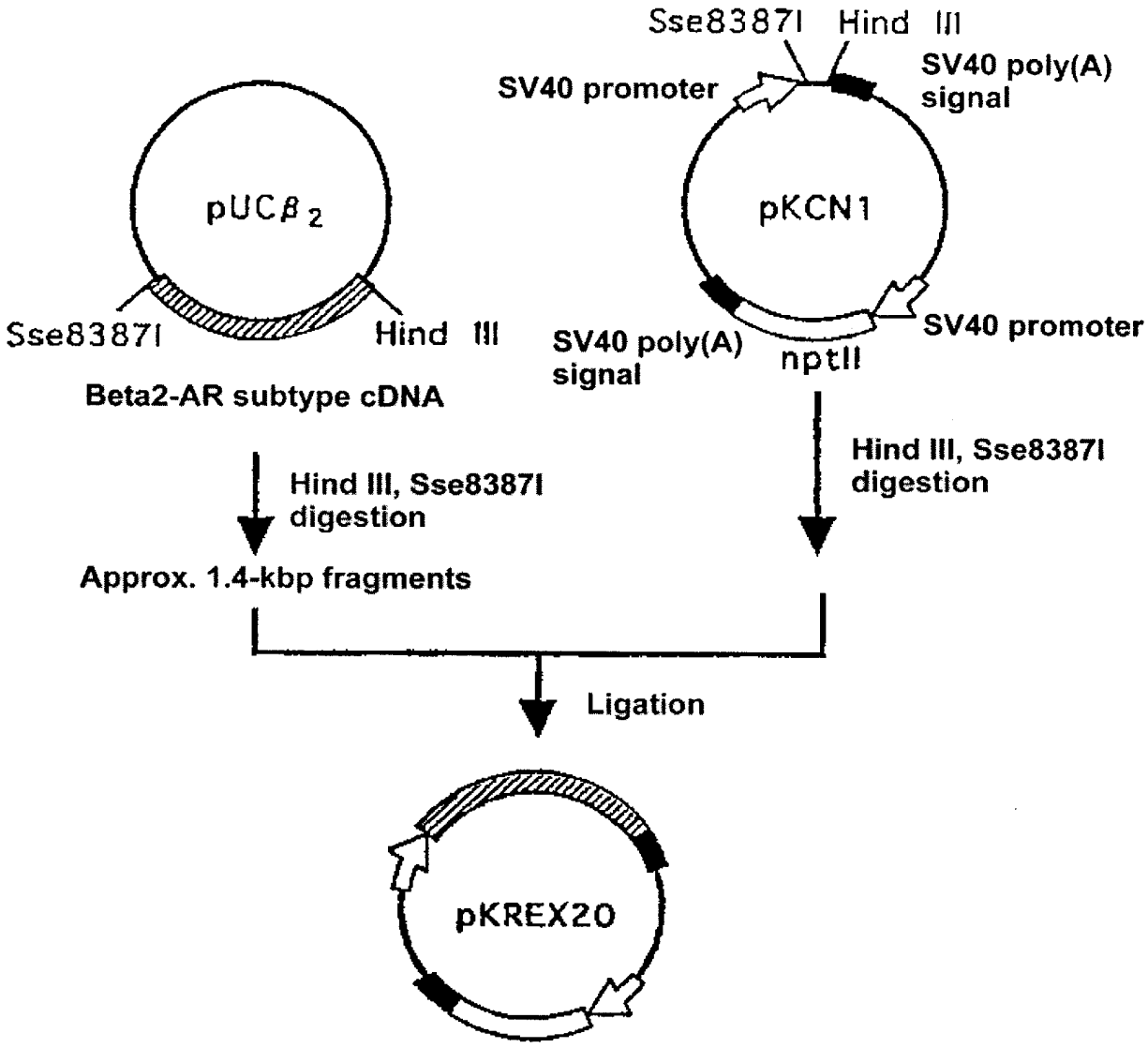

Sse8387I
Hind III
SV40 poly(A) signal
SV40 promoter
pKCN1
pUCβ2
Sse8387I
Hind III
SV40 poly(A) signal
nptII
SV40 promoter
Beta2-AR subtype cDNA
Hind III, Sse8387I digestion
Hind III, Sse8387I digestion
Approx. 1.4-kbp fragments
Ligation
pKREX20

Figure 6

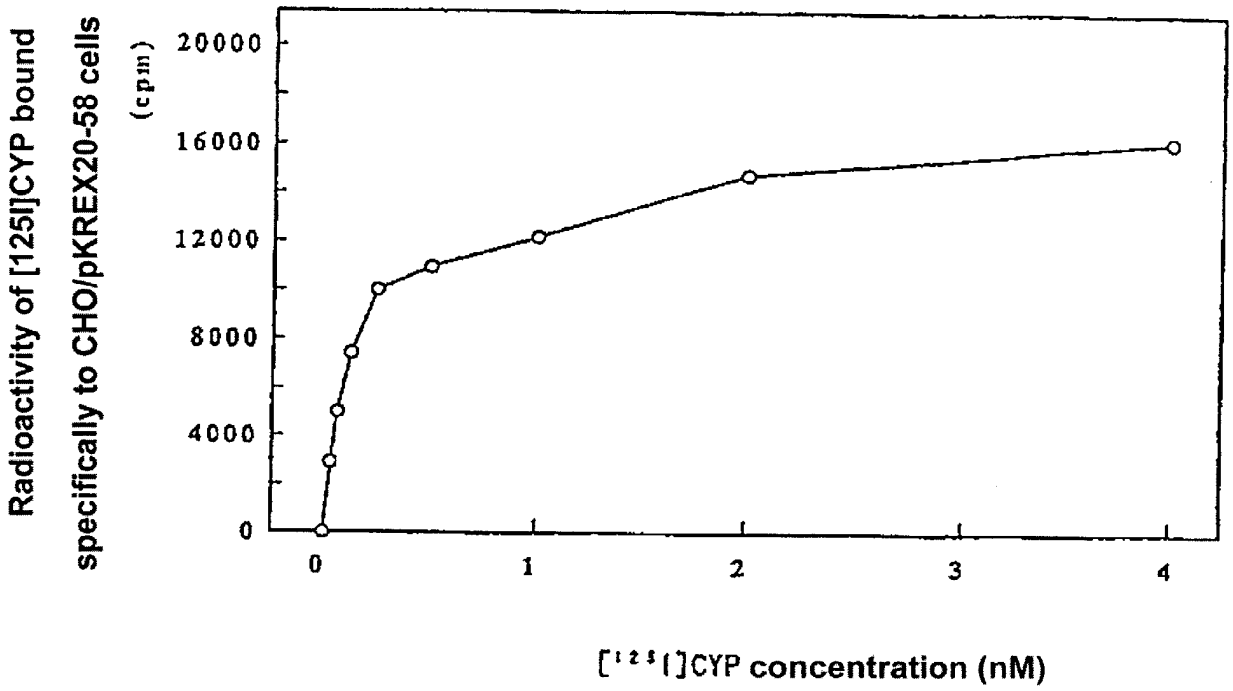

Radioactivity of [125I]CYP bound specifically to CHO/pKREX20-58 cells
(cpm)
20000
16000
12000
8000
4000
0
0
1
2
3
4
[125I]CYP concentration (nM)

Figure 7

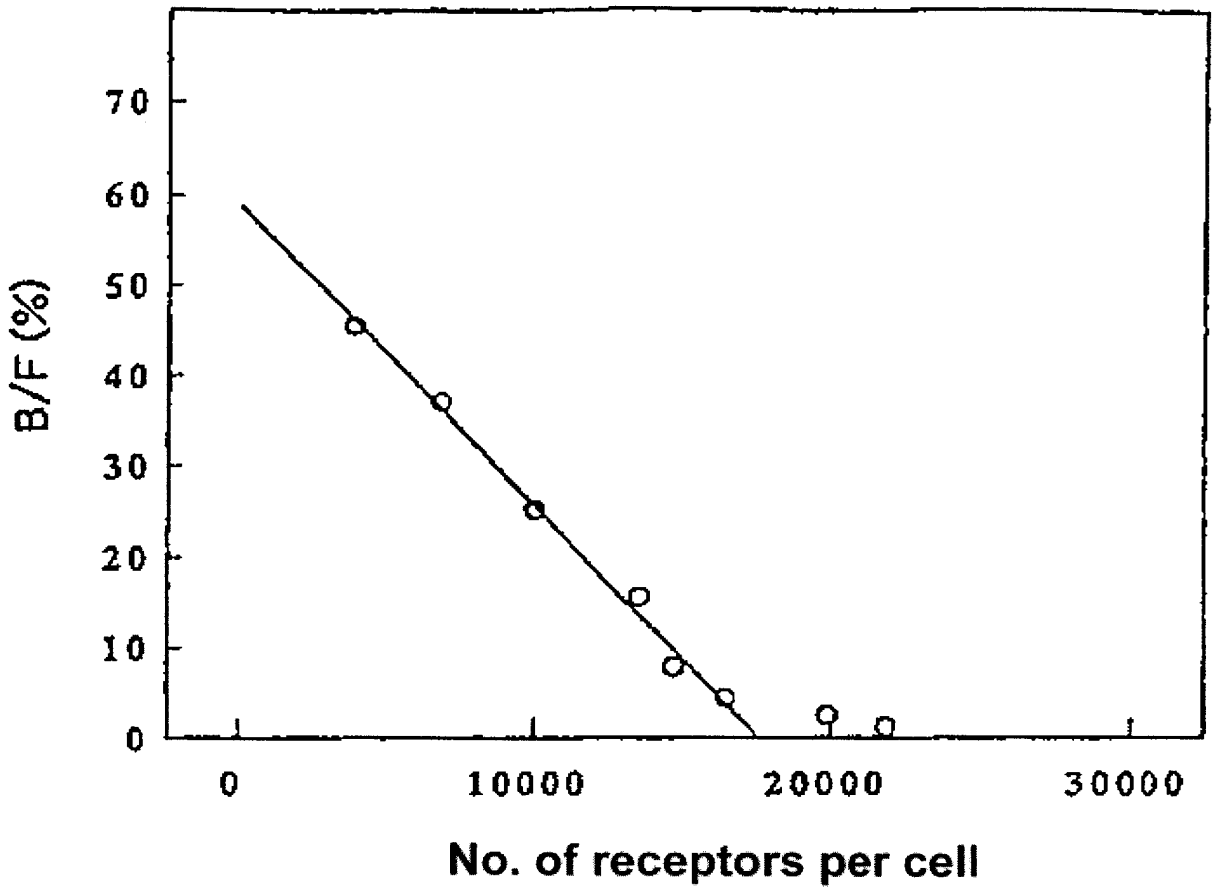

B/F (%)
70
60
50
40
30
20
10
0
0
10000
20000
30000
No. of receptors per cell

Figure 8

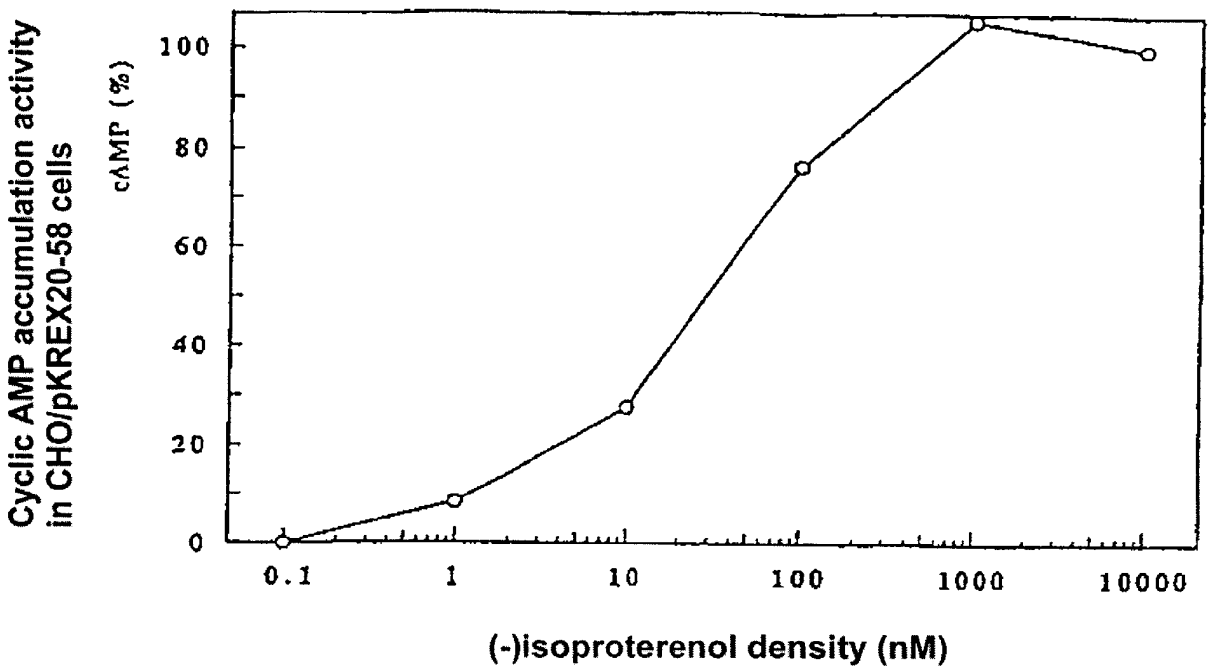
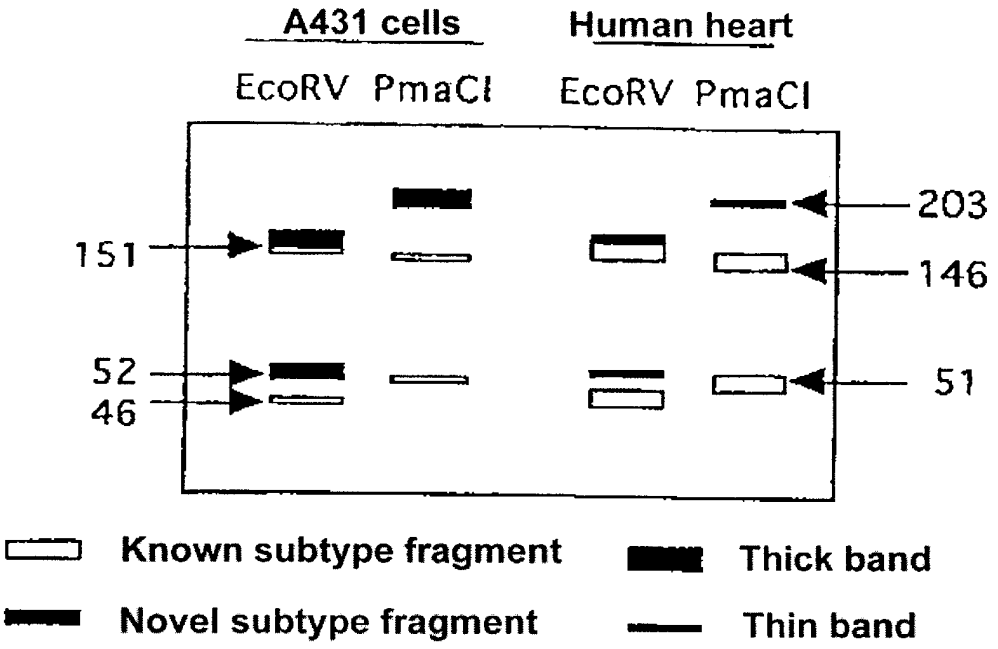
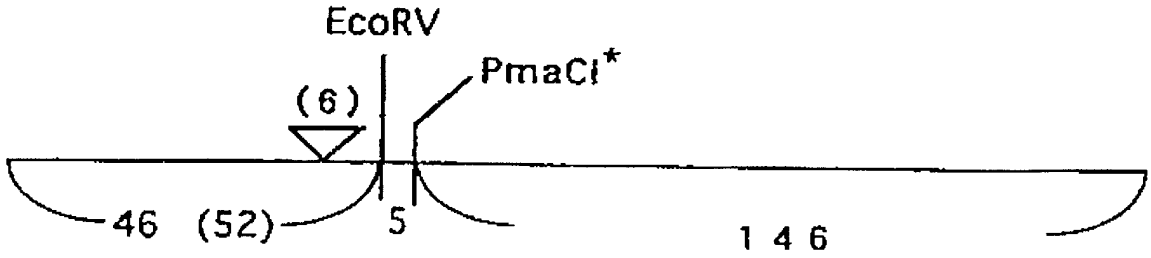

Cyclic AMP accumulation activity in CHO/pKREX20-58 cells
cAMP (%)
100
80
60
40
20
0
0.1
1
10
100
1000
10000
(-)isoproterenol density (nM)

Figure 9

(a)



(b)



* Digested in fragments derived from novel subtype